IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Oreste, *et al.*
Serial No.: 10/518,302
Filed: June 17, 2003
Title: EPIMERIZED DERIVATIVES OF K5 POLYSACCHARIDE WITH A VERY HIGH DEGREE OF SULFATION

Group Art Unit: 1623
Examiner: Layla D. Bland

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION PURSUANT TO 37 C.F.R. §1.132

Sir:

I, Pasqua ORESTE, a biochemist graduated in Biology at the University of Milan (Italy) do hereby declare as follows:

1. I am joint inventor of the instant application and jointly invented the whole subject matter claimed therein together with the other inventor, Giorgio Zoppetti. I am familiar with the instant application and have read the Office Action mailed on March 2, 2010 as well as the cited references that I know very well.
2. I have also personally carried out and presently carry out chemical reactions generally in the glycosaminoglycan chemistry and particularly regarding sulfation of epimerized and non-epimerized K5-N-sulfates.
3. I am also familiar with literatures regarding polysaccharide K5 and its semisynthetic derivatives, in particular with the Casu et al application WO 98/42754 (Casu) cited by the Examiner. In addition, I am one of the Authors, together with the other inventor, Giorgio Zoppetti, of the the Leali et al. paper (Leali) also cited by the Examiner, as well as joint inventor, together with the other inventor, Giorgio Zoppetti, of US 6,992,183 ("US patent") disclosing the non-epimerized N,O-oversulfated K5-polysaccharide used by Leali et al.
4. All of the Casu, US Patent (and Leali) and instant application disclose an oversulfation of an epimerized, N-sulfated K5 polysaccharide (epiK-5-N-sulfate) or of a non-epimerized. N-sulfated K5 polysaccharide (K5-N-sulfate), herein below generically referred to as "substrate", by a reaction consisting of the treatment of an epi-K5-N-sulfate or K5-N-sulfate tertiary amine or quaternary ammonium salt (in particular the tetrabutylammonium ["TBA"] salt) with an

excess of the complex pyridine.SO_3 in dimethylformamide, i.e. under the oversulfation conditions [C], described by Casu et al in Carbohydrate Research 1994, 266, 271-284 ("Casu 1994") and carried out on heparin or K5-N-sulfate.

5. The preparation and isolation of the TBA salt of the substrate, by the addition of tetrabutylammonium hydroxide ("TBA-OH") to the aqueous solution containing said substrate in acidic form, are as follows:
   - Casu 1994 adjustes the pH to 5.5 by addition of TBA-OH in ethanol, then lyophilizes the obtained TBA salt solution to isolate said salt; the maximal attained sulfation degree, after N-resulfation, is 3.1
   - Casu brings the pH to 9 by addition of TBA-OH in ethanol, then lyophilizes the obtained TBA salt solution to isolate said salt;
   - US Patent (or Leali) brings the pH to 7 by addition of TBA-OH in water, then concentrates the solution and lyophilizes to isolate the TBA salt;
   - instant application brings the pH to 7 by addition of TBA-OH in water, then wait (for 30-60 minutes) by continuously adjusting the pH (which decreases) to 7 by addition of TBA-OH, then lyophilizes the obtained TBA salt solution to isolate said salt.

To clarify the differences between the the TBA salt formation in the methods of oversulfation described above, we decided to reproduce Example 4(a) and (b) under my supervision by performing the oversulfation process after the passage of an aqueous solution of the substrate through an IR 120 H^+ resin by adding TBA-OH in water under the following different conditions: to pH 7 and waiting 1 hour at pH 7 before lyophilization and oversulfation according to instant application (SAMPLE 1), to pH 7 and subsequent lyophilization and oversulfation as described by Leali et al (SAMPLE 2) and, respectively, to pH 9.0 and subsequent lyophilization and oversulfation under the conditions of Casu (SAMPLE 3). After the oversulfation, the ^{13}C-NMR spectra of the oversulfated products (epiK5-amine-O-oversulfates) were performed, the amount of sulfate groups in the different positions of the disaccharide were calculated and compared with the amount of sulfate groups in the corresponding positions of the disaccharide calculated from the ^{13}C-NMR of the K5-amine-O-oversulfate intermediate (referred to as "non-epimerized" or "K5 non epi") of the K5-N,O-oversulfate of US Patent (or Leali) currently prepared by carrying out the TBA salt formation as described in the present application, i.e. by maintaining pH 7 for about one hour by addition of TBA-OH (see "Experimental" below)

Experimental

1. Preparation of epiK5-amine-O-oversulfate

(a) Tetrabutylammonium salt of epiK5-N-sulfate

In three different preparation, a solution in 40 ml of water of 400 mg of epiK5-N-sulfate was thermostated at 4°C, then passed over IR 120^+ ionic exchange resin preconditioned with water at 4°C. The eluate obtained, consisting of 100 ml of a solution at pH 1.94 was treated as follows:

(1) the solution containing epiK5-N-sulfate in acidic form was neutralized (pH 7) with a 15% solution of tetrabutylammonium (TBA) hydroxide and left at room temperature for one hour, maintaining the pH at 7 by addition of 15% tetrabutylammonium hydroxide and finally was lyophilized to give TBA-epiK5-N-sulfate(1);

(2) the solution containing epiK5-N-sulfate in acidic form was neutralized (pH 7) with a 15% solution of tetrabutylammonium hydroxide, then concentrated to small volume and lyophilized (Leali et al) to give TBA-epiK5-N-sulfate(2).

(3) the solution containing epiK5-N-sulfate in acidic form was brought to pH 9 with a 15% solution of tetrabutylammonium hydroxide, then lyophilized (Casu et al) to give TBA-epiK5-N-sulfate(3).

(b) EpiK5-amine-O-oversulfate

In three different preparations, a solution in 30 ml of dimethylformamide containing the TBA salt (1), (2) and, respectively, (3) thus obtained was set at 55°C and treated with 30 ml of dimethylformamide containing 2.26 g of pyridine.SO_3 adduct. The reaction at 55°C was continued overnight, then 60 ml of water were added to the mixture. After neutralization with 1N NaOH, the product was precipitated with 3 volumes of acetone saturated with NaCl and set at 4°C overnight. The precipitate was recovered by filtration on guch G4, then ultrafiltered with 1000 D TFF Millipore system and dried at reduced pressure to give

SAMPLE 1: epiK5-amine-O-oversulfate from TBA-epiK5-N-sulfate(1) from TBA salt (1),

SAMPLE 2: epiK5-amine-O-oversulfate from TBA-epiK5-N-sulfate(2) from TBA salt (2), and, espectively,

SAMPLE 3: epiK5-amine-O-oversulfate from TBA-epiK5-N-sulfate(3) from TBA salt (3).

2. Preparation of K5-amine-O-oversulfate ("Non-epimerized").

(a) Tetrabutylammonium salt of K5-N-sulfate

A solution in 40 ml of water of 400 mg of K5-N-sulfate was thermostated at 4°C, then passed over IR 120^+ ionic exchange resin preconditioned with water at 4°C. The eluate obtained, consisting of 100 ml of a solution at pH 1.94 was treated as follows:

(1) the solution containing K5-N-sulfate in acidic form was neutralized (pH 7) with a 15% solution of tetrabutylammonium (TBA) hydroxide and left at room temperature for one hour,

maintaining the pH at 7 by addition of 15% tetrabutylammonium hydroxide and finally was lyophilized to give TBA-K5-N-sulfate

(b) K5-amine-O-oversulfate

A solution in 30 ml of dimethylformamide containing the TBA salt thus obtained was set at 55°C and treated with 30 ml of dimethylformamide containing 2.26 g of pyridine.SO_3 adduct. The reaction at 55°C was continued overnight, then 60 ml of water were added to the mixture. After neutralization with 1N NaOH, the product was precipitated with 3 volumes of acetone saturated with NaCl and set at 4°C overnight. The precipitate was recovered by filtration on guch G4, then ultrafiltered with 1000 D TFF Millipore system and dried at reduced pressure to give K5-amine-O-oversulfate ("Non –epimerized").

The ^{13}C-NMR spectra of the SAMPLE 1, SAMPLE 2, SAMPLE 3 and Non-epimerized products are attached herewith. The Table below reports the sulfation profile of each of the above four products as determined by interpretation of the four attached spectra.

Table

	SAMPLE 1 pH 7 1 h	**SAMPLE 2** pH 7	**SAMPLE 3** pH 9	**Non-epimerized pH7 1 h**
A6S	1	0.9	0.8	1
A3S	0.7	0.3	0.30	0.37
G3S	0.0	0.35	0.35	0.72
I3S	0.1	0.2	0.2	
U0S	0.0	0.60	0.35	0.0
UdiS	0.9	0.2	0.45	0.35
Sulfate/carboxyl	3.6	2.15	2.55	2.79

A = glucosamine, G = glucuronic acid, I = Iduronic acid, U = Uronic acids indifferently, NS = N-sulfate.

In this Table, the numbers in the column refer to the sulfation degree (sulfate/carboxyl ratio) per disaccharide unit (1 = 100% sulfation), the unsulfated uronic acids, U0S (UzeroS), are not computed in the calculation of the sulfation degree and the disulfated uronic units, UdiS, must be doubled in said calculation.

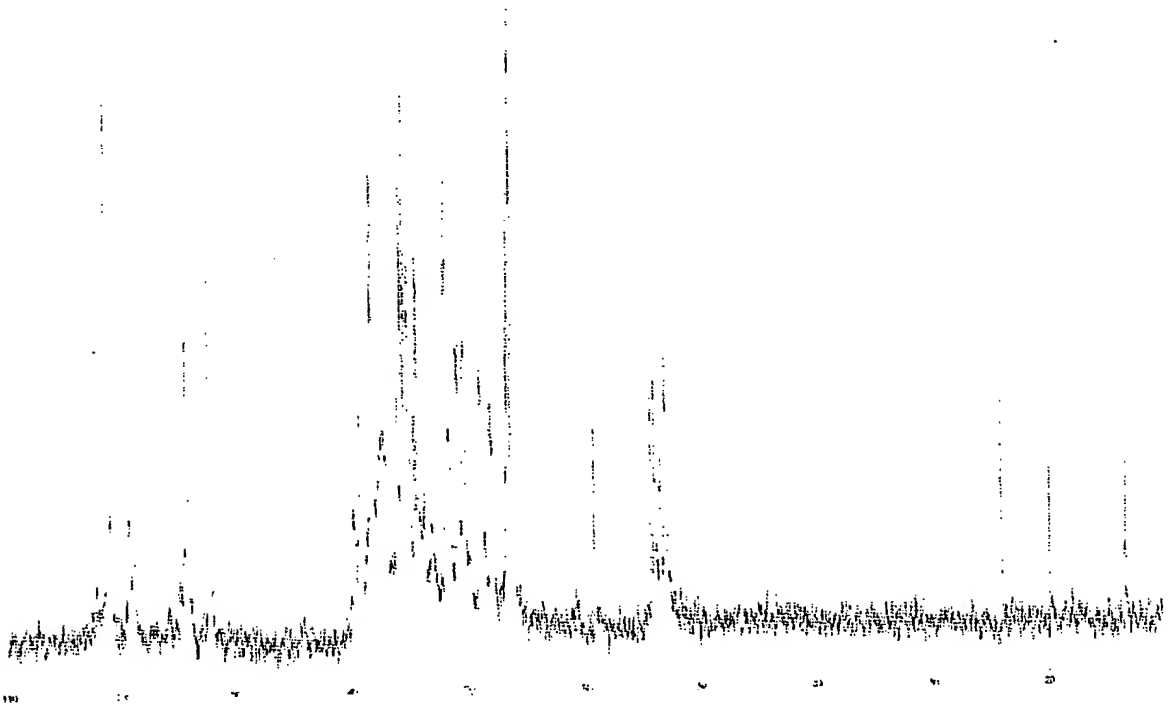
6. The result illustrated in the Experimental confirms that an (only apparently) little modification of the currently used oversulfation method a very highly sulfated [sulfation degree of 3.6, very close to that of Example 4 (b)] epiK5-amine-O-oversulfate, characterized by a very high content in glucosamine 3-O-sulfate (70%), can be obtained, while, by operating under the same conditions as those of the present application, the sulfation degree (2.79) of the non-epimerized amine product, even though higher than that of those of the reference epiK5-amine-O-oversulfates, is much lower of that of the corresponding epimerized amine and shows a glucosamine 3-O-sulfate content lower than 40%.

7. The results illustrated in the Experimental show that by oversulfating an epimerized K5-N-sulfate under the conditions of our invention, an epiK5-amine-O-oversulfate characterized by a very high content in glucosamine 3-O-sulfate (70%) and having a sulfation degree [3.6, very close to that of Example 4 (b)] much higher than that of the epiK5-amine-O-oversulfates prepared under the conditions known at the time of our invention are obtained. Said results also show that by operating under the same conditions as those of the present application, the sulfation degree (2.79) of the non-epimerized amine product, even though higher than that of those of the reference epiK5-amine-O-oversulfates, is much lower than that of the corresponding epimerized amine and has a glucosamine 3-O-sulfate content lower than 40%.
8. I conclude that, of my knowledge, the sole way for reaching a degree of sulfation higher than 4 in an epiK5-N,O-oversulfate-derivative by oversulfation of an epiK5-N-sulfate and final N-sulfation is that opened by our, presently claimed invention.
9. I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that the making of willful false statements and the like are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful statements may jeopardize the validity of the applications or any patent issuing thereon.

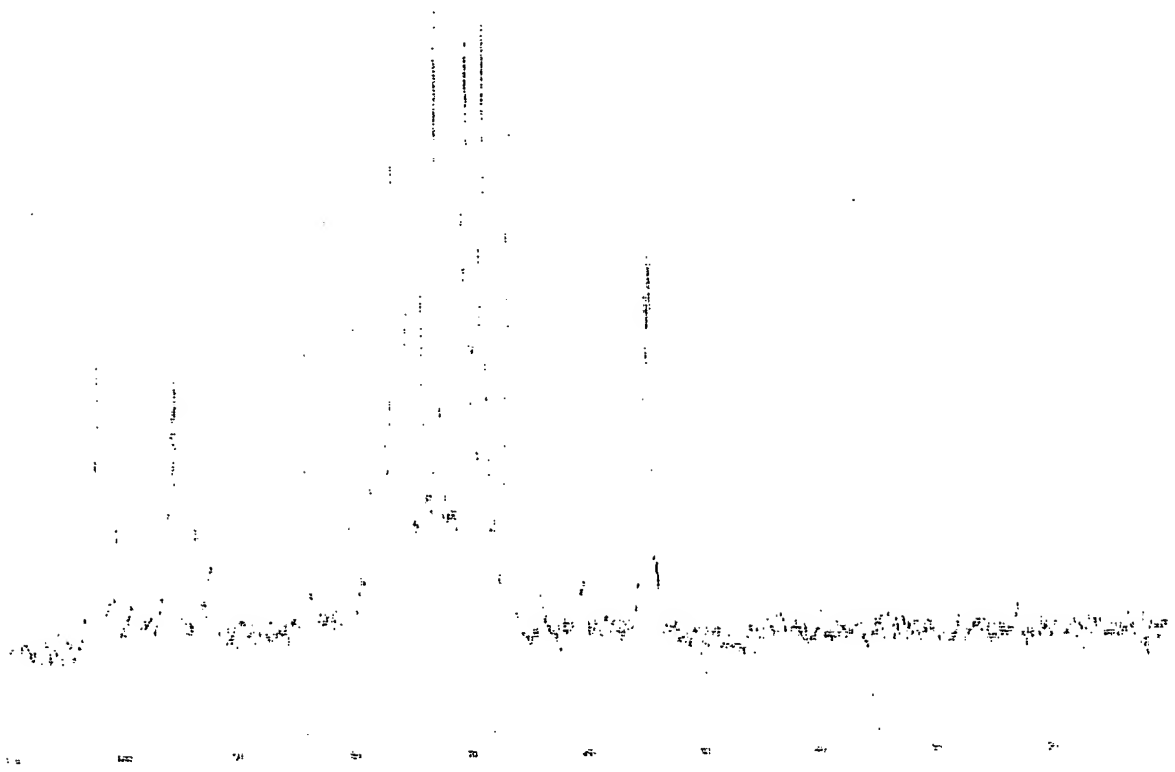
Dated: 01/06/2010

Pasquale Oreste

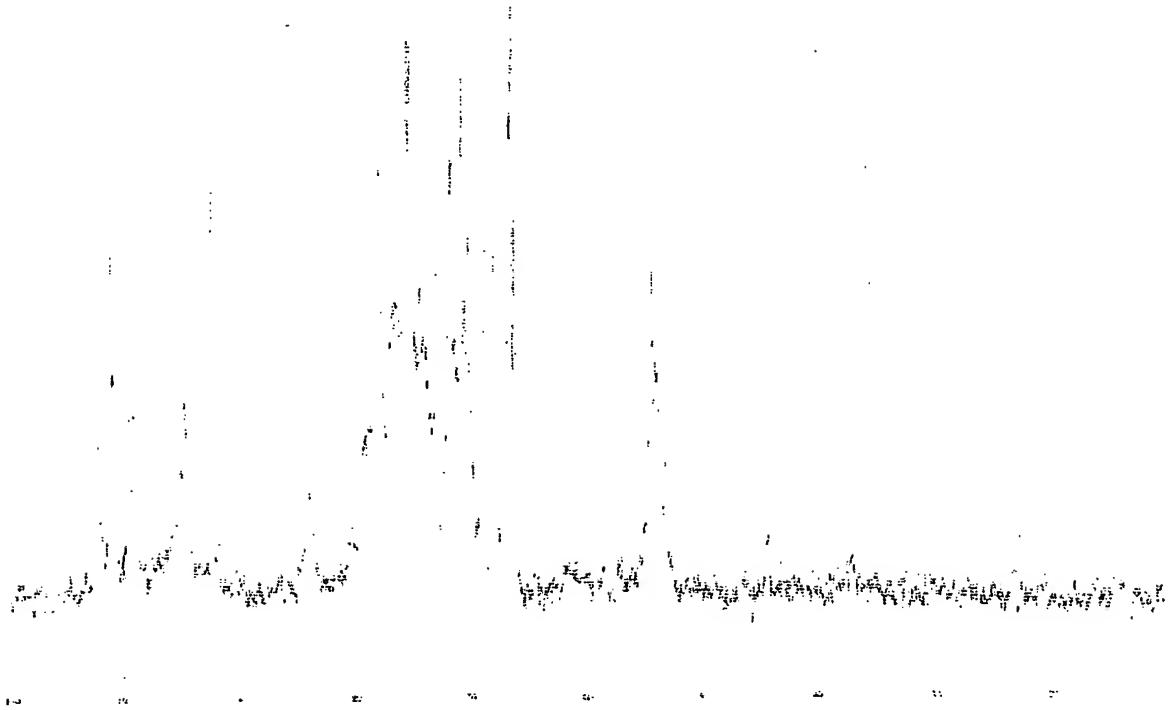
Pasquale ORESTE



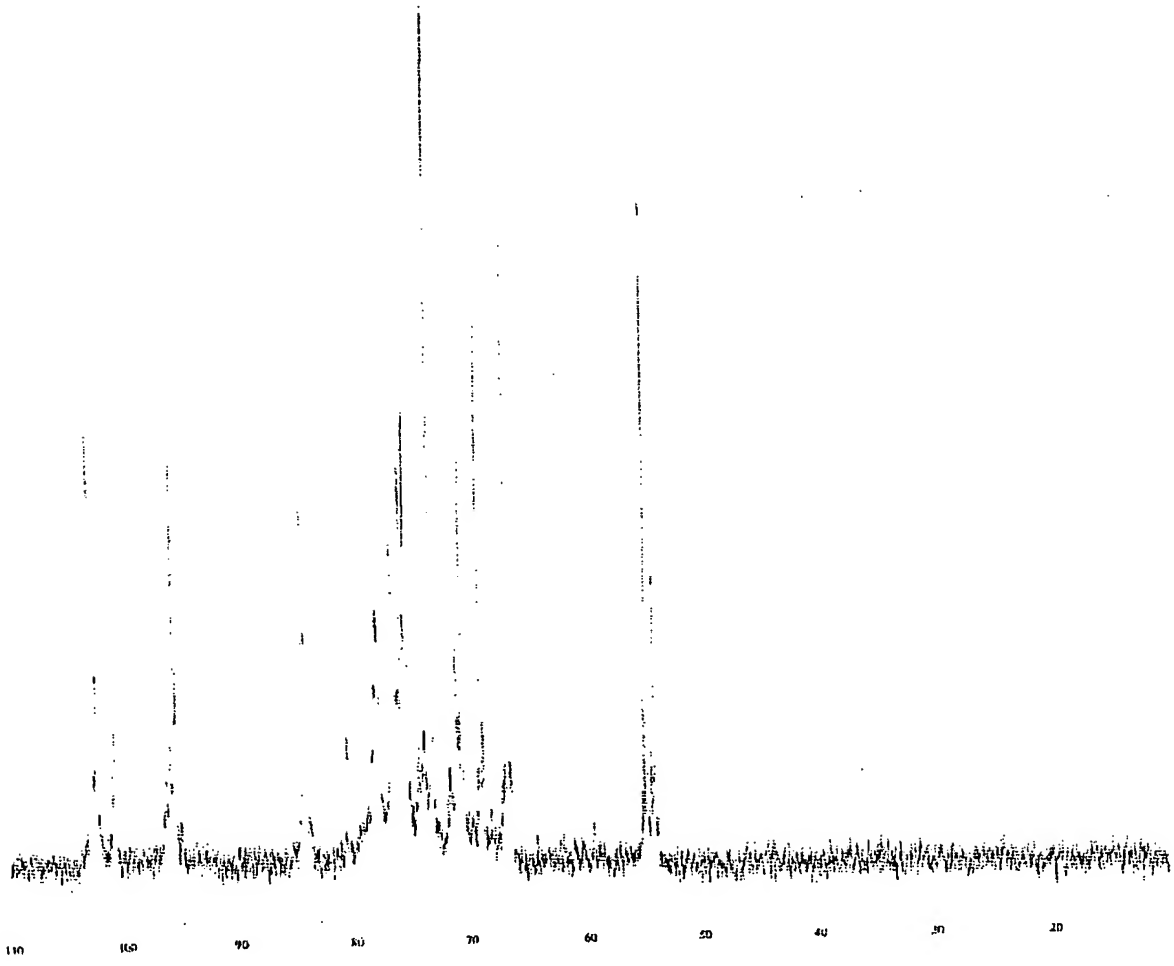
SAMPLE 1



SAMPLE 2



SAMPLE 3



K5 non-epi